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Clostridium perfringens β-toxin is sensitive to thiol-group modification but does not require a thiol group for lethal activity

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Abstract

The β-toxin gene isolated from *Clostridium perfringens* type B was expressed as a glutathione *S*-transferase (GST) fusion gene in *Escherichia coli*. The purified GST-β-toxin fusion protein from the *E. coli* transformant cells was not lethal. The N-terminal amino acid sequence of the recombinant β-toxin (r toxin) isolated by thrombin cleavage of the fusion protein was G-S-N-D-I-G-K-T-T-T. Biological activities and molecular mass of r toxin were indistinguishable from those of native β-toxin (n toxin) purified from *C. perfringens* type C. Replacement of Cys-265 with alanine or serine by site-directed mutagenesis resulted in little loss of the activity. Treatment of C265A with *N*-ethylmaleimide (NEM), which inactivated lethal activity of r toxin and n toxin, led to no loss of the activity. The substitution of tyrosine or histidine for Cys-265 significantly diminished lethal activity. In addition, treatment of C265H with ethoxyformic anhydride which specifically modifies histidyl residue resulted in significant decrease in lethal activity, but that of r toxin with the agent did not. These results showed that replacement of the cysteine residue at position 265 with amino acids with large size of side chain or introduction of functional groups in the position resulted in loss of lethal activity of the toxin. Replacement of Tyr-266, Leu-268 or Trp-275 resulted in complete loss of lethal activity. Simultaneous administration of r toxin and W275A led to a decrease in lethal activity of β-toxin. These observations suggest that the site essential for the activity is close to the cysteine residue.

Keywords: β-Toxin; Glutathione *S*-transferase fusion; Thiol group; Site-directed mutagenesis; (*Clostridium perfringens*)

1. Introduction

Clostridium perfringens β-toxin is thought to be the significant agent in the causation of necrotic enteritis caused by the type C strains, which is characterized by hemorrhagic mucosal ulceration or superficial mucosal necrosis of the small intestine [1–3]. We have reported the extensive purification of β-toxin produced by *C. perfringens* type C and some of the physicochemical properties of the toxin [4–6]. We reported the toxin acts on the autonomic nervous system and produces arterial constriction [7,8]. We also reported that the toxin is inhibited by sulfhydryl group reagents and oxidizing agents, the toxin treated with *p*-chlormercuribenzoate (PCMB) and oxidizing agents is reactivated by reduced glutathione and reductants, respectively, and that the number of thiol group is one per mol of β-toxin from *C. perfringens* [9,10]. The β-toxin gene from *C. perfringens*

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has been cloned and sequenced [11]. The deduced amino acid sequence showed that the toxin contains only one cysteine residue. These observations have led to the belief that the sulfhydryl group plays a critical role in the toxin activity. However, the precise role of the cysteine residue plays in toxin activity is unclear.

Expression of the cloned β-toxin gene in *Escherichia coli* resulted in production of high molecular mass protein [11]. The β-toxin showed a polymeric form, which is inactive. Furthermore, Steinthorsdottir et al. [12] reported that treatment of the GST-β-toxin fusion protein from the gene constructed using pGEX-3X vector with Factor Xa caused no activity of the toxin. Therefore, we tried to construct the GST-β-toxin gene using pGEX-4T-1 vector and isolate the active form of r toxin from GST-β-toxin fusion protein. Furthermore, we replaced the single cysteine residue and amino acid residues near the cysteine residue with other amino acids by site-directed mutagenesis, and examined lethal activity of variant β-toxins constructed.

2. Materials and methods

2.1. Purification of β-toxin from C. perfringens type C

Procedures for culture of *C. perfringens* type C (CN5386) and purification of β-toxin were described previously [4,6].

2.2. Bacterial strains and plasmid

C. perfringens type B (NCTC 8533) and *E. coli* JM109 were used as bacterial strains. pT7-Blue vector (Novagen) was used for cloning and pGEX-4T-1 (Pharmacia) was used for cloning and expression of the β-toxin gene.

2.3. Preparation of DNA

C. perfringens was anaerobically cultured at 37°C in brain heart infusion broth. Chromosomal DNA was isolated from the bacteria used in this study according to the method described previously [11]. DNA extracted from *C. perfringens* was purified by centrifugation through a gradient of cesium chloride [13]. Large- and small-scale plasmid isolation were performed as described by Sambrook et al. [13].

2.4. PCR amplification and cloning

C. perfringens DNA encoding the β-toxin gene was amplified by using the polymerase chain reaction (PCR) with a set of primers β-1 (5′-TTTAACTTAACAGATCATCTATA-3′) and β-2 (5′-ACTAATTTGAAATTCATATTAGT-3′), selected on the basis of nucleotide sequence described [11]. The PCR was performed for 25 cycles at 94°C for 1 min, 45°C for 30 s and 72°C for 2 min. The PCR products were gel-purified, and ligated into pT7-Blue vector (plasmid pTB-1). Plasmid pTB-1 was used as a template for PCR to add a *Bam*HI site for subcloning into the vector using a primer 5′-GGGGATCCAATGATATAGGTAAAC-3′ (*Bam*HI site is underlined) and a reverse primer. The product was isolated, digested with *Bam*HI and *Sal*I, and ligated into a pGEX vector previously digested with the same enzymes so that the correct reading frame was maintained with thrombin cleavage site under the GST gene. However, the r toxin contained two non-native amino acids, Gly–Ser, added to the N-terminus as a result of thrombin cleavage.

2.5. Site-directed mutagenesis

Site-directed mutagenesis was performed by the unique restriction enzyme site elimination technique using the Transformer mutagenesis kit (Clontech Lab., Palo Alto, CA, USA) with synthetic appropriate oligonucleotide primers as described previously [14,15]. Plasmid pTB-1 was used as a template for mutagenesis. All mutants obtained were identified by sequencing with the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems), as described previously [14,15]. The variant toxin genes were ligated into a pGEX vector as described above. The variant toxins constructed under the condition contain two non-native amino acids, Gly–Ser, added to the N-terminus.

2.6. Expression and purification of wild-type and variant toxins

A 2-ml culture of L-broth containing 100 μg/ml of

ampicillin was inoculated with a single recombinant colony of *E. coli* JM 109 bearing the desired construct and incubated overnight at 37°C with shaking. One ml of this culture was then used to inoculate larger cultures (100 ml of medium composed of tryptone (Difco) 16 g, yeast extract (Difco) 10 g, NaCl 5 g and 2% glucose/l) and the resulting culture was incubated at 37°C, with shaking at 300 rpm until reaching an OD_{600} of 0.8–0.9. At this point, fusion protein was induced by adding filtered IPTG to 1 mM, and the culture was allowed to grow with shaking at 300 rpm for 2 h. The culture was then centrifuged at $4000 \times g$ at 4°C for 20 min. The supernatant was decanted, and the cell pellet was suspended in 10 ml of buffer A (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and the suspension was disrupted by sonicator on ice in short burst. The lysate was clarified by centrifugation at $40\,000 \times g$ at 4°C for 30 min. The supernatant of the lysate was applied directly to glutathione–Sepharose column (Pharmacia). After application of the lysate, the column was washed with 10 column volumes of buffer A. To the column was added the thrombin solution (10 units) in buffer A to enter into the bed of Sepharose and the bottom of the column was capped, and the column allowed to stand at room temperature for 16 h. Following incubation, the column was eluted with buffer A to elute protein. The protein fraction was chromatographed on a Mono Q column (Pharmacia), previously equilibrated with 0.02 M Tris–HCl buffer (pH 8.0), and wild-type or variant toxins were separated from thrombin.

2.7. Production of polyclonal mouse antisera

Anti-β-toxin antiserum was prepared by immunizing mice (about 20 g) with a toxoid obtained from n toxin by treatment with 0.4% formalin at 37°C for 2 days. Freund's complete adjuvant was mixed with the antigen, and intrasubcutaneous injections (containing 10 μg of protein) were given at 2-week intervals. Blood was collected 10 days after immunization.

2.8. SDS–PAGE and Western blot

The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in gels containing 12.5% polyacrylamide and visualized by Coomassie brilliant blue. For Western blots, proteins were transferred by electroplating onto polyvinylidene difluoride membrane (Immobilon P, Millipore). The membrane first was incubated with polyclonal anti-n toxin (mouse) antiserum (diluted 1:1000) and then with anti-mouse IgG-peroxidase conjugate (diluted 1:10 000) (Amersham). Wild-type and variant toxins on the membrane were visualized by the enhanced chemiluminescence Western blotting analysis system (Amersham) [14,15].

2.9. Chemical modification

β-Toxin and variant toxins were modified by NEM, sulfhydryl group reagent [9,10] or ethoxyformic anhydride (EFA), histidine residue modifying agent [16] as described previously.

2.10. Biological activity

Male mice of the ddY strain, weighing 20–25 g, were used. Toxicity was determined by i.v. injection of serial dilutions (0.1 ml of the toxin solution) into groups of four mice (20–25 g). Deaths occurring within 24 h were recorded.

2.11. Others

The N-terminal amino acid sequence of the toxin was determined as described previously [11] using a protein sequencer (473A, Applied Biosystems). Circular dichroism (CD) spectra were recorded with a spectropolarimeter (Jasco, J-500C, Tokyo, Japan) as described previously [17]. Protein contents were assayed by the method described by Lowry et al. [18].

3. Results

3.1. Purification and lethal activity of r toxin from GST-β-toxin fusion protein

The supernatant fluid of the sonicated *E. coli* transformant cells was analyzed by Western blotting using antisera against GST and n toxin. A single

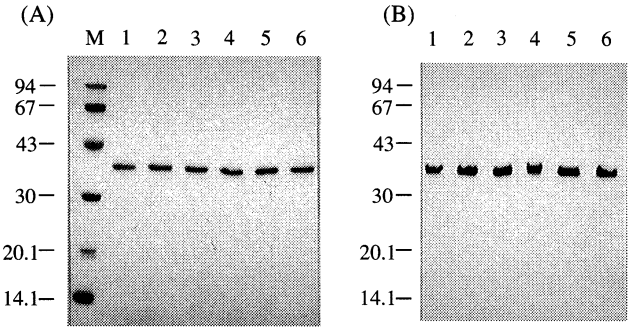


Fig. 1. SDS–PAGE and Western blot of native β-toxin, recombinant β-toxin and variant β-toxins. (A) The purified preparation (5 μg of protein) was electrophoresed by SDS–PAGE (12.5% polyacrylamide gel) followed by staining 1% (w/v) Coomassie brilliant blue. (B) The purified preparation (0.5 μg of protein) was electrophoresed and subsequently electroblotted to PVDF membrane (Millipore). The immunoblot analysis were performed with anti-n toxin antitoxin as described in the text. Lane assignments: M, molecular mass marker: lane 1, n toxin purified from culture of *C. perfringens*: lane 2, r toxin: lane 3, C265A: lane 4, C265S: lane 5, C265H: lane 6, C265Y.

band of about 62 kDa was detected by anti-n toxin antiserum, suggesting that GST-β-toxin fusion protein was produced by the *E. coli* transformant cells. After application of supernatant fluid of the sonicated cells to glutathione–Sepharose affinity column, the thrombin solution was applied to the column. The column stood at room temperature for 16 h and then r toxin was eluted with the buffer. To separate r toxin and thrombin, the toxin fraction was loaded onto a Pharmacia fast-performance liquid chromatography Mono-Q HR55 column preequivalent with 20 mM Tris–HCl (pH 8.0) and the column was eluted with a 25 ml of gradient from 0 to 1 M NaCl in 20 mM Tris–HCl (pH 8.0). The toxin was eluted as a sharp peak at a chloride concentration of about 200 mM (data not shown). The purity of the preparation was tested by SDS–PAGE. Fig. 1 shows that a single band of about 35 kDa was observed by Coomassie brilliant blue staining and the mobility of the preparation was identical to that of n toxin. We analyzed the N-terminal amino acid sequence of the purified preparation using an automated protein sequencer. The amino acid sequence was G-S-N-D-I-G-K-T-T-T (data not shown). Therefore, it is apparent that the r toxin contains an additional Gly–Ser linked to the N-terminus of n toxin, judging from that obtained by nucleic acid sequencing of the β-toxin gene [11]. The yield of the r toxin was about 1 mg/l of culture.

To determine the effect of the additional two amino acids linked to the N-terminus of r toxin on lethal activity of β-toxin, the activity of r toxin and n toxin was tested in mice. As shown in Table 1, a dose of 30 ng of n toxin or r toxin per mouse killed them within 24 h, but when given 15 ng of these toxins, no death occurred. The result shows that lethal activity of r toxin was as active as that of n toxin, indicating that the addition of the two amino acids causes no effect on the activity of β-toxin.

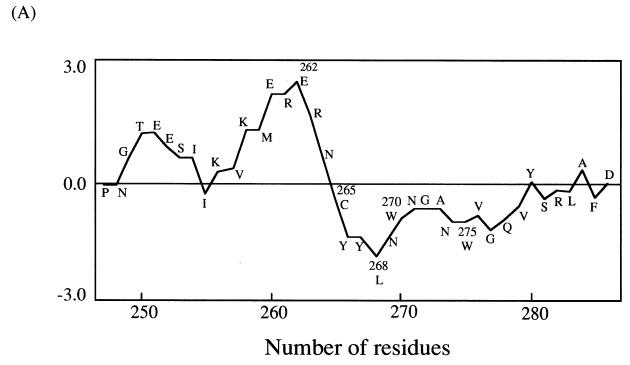
3.2. Production and purification of variant β-toxins

To clarify the role of the Cys-265 residue and amino acid residues (Glu-262, Tyr-266, Leu-268, Trp-270 and Trp-275) near the cysteine residue in the toxin, site-directed mutagenesis was employed to convert these amino acid codons to one for alanine, glycine, histidine, phenylalanine, serine or tyrosine. On the constructed plasmids, modifications of the Cys codon and these amino acid codons of the β-toxin gene were identified by nucleic acid sequencing (data not shown). Putative variant β-toxin genes were expressed as a GST-variant β-toxin fusion proteins in *E. coli*. According to the purification procedure of r toxin, GST-β-toxin fusion proteins were purified from the sonicate of these *E. coli* transformant cells and variant β-toxins were isolated by treatment of

Table 1
Lethal activity of native, recombinant and variant β-toxins

Toxin	Dose of toxin (ng/mouse)								
	3840	1920	960	480	240	120	60	30	15
	(No. of mice death/No. of mice injected)								
n beta-toxin						4/4	4/4	4/4	0/4
r beta-toxin						4/4	4/4	4/4	0/4
C265A						4/4	4/4	3/4	0/4
C265S						4/4	4/4	3/4	0/4
C265H			4/4	4/4	0/4	0/4			
C265Y			4/4	4/4	1/4	0/4			
E262A						4/4	4/4	4/4	0/4
Y266A	0/4	0/4	0/4						
L268G	0/4	0/4	0/4						
W270F						4/4	4/4	3/4	0/4
W275A	0/4	0/4	0/4						

Native, recombinant or variant β-toxin was injected into mice intravenously. Deaths occurring within 24 h were recorded.



(B)

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          .   *   *     : * .  :     *   :   * .  .   : *   *     .
Beta  253 S-I-I-K-V-K-M-E-R-E-R-N-C-Y-Y-L-N-W-N-G-A-N-W-V-G-Q-V-Y 280
Hla   243 T-N-I-D-V-I-Y-E-R-V-R-D-D-Y-Q-L-H-W-T-S-T-N-W-K-G-T-N-T 270
LukF  237 S-K-I-T-V-T-Y-Q-R-E-M-D-L-Y-Q-I-R-W-N-G-F-Y-W-A-G-A-N-Y 264
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Fig. 2. (A) Predicted hydropathy of β-toxin. The hydropathic potentials of β-toxin (247–286 residues) were predicted by the method of Hopp and Woods [31]. Positive values within the plot indicate hydrophilicity and negative values indicate hydrophobicity. (B) Alignment of deduced amino acid sequences of *C. perfringens* β-toxin and *S. aureus* α-toxin (Hla) and fast component of leucocidine (Luk F). (*) Identical residues with all toxins; (:) identical residues with β-toxin and Hla; (.) identical residues with β-toxin and Luk F.

Table 2
Effect of *N*-ethylmaleimide and ethoxyformic anhydride on lethal activity of recombinant β-toxin, C265A and C265H

Toxin	Agent	Dose of toxin (ng/mouse) 3840	1920	960	480	240	120	60	30	15
		(No. of mice death/ No. of mice injected)								
Experiment 1[a]										
r beta-toxin	NEM 0 mM							4/4	4/4	0/4
r beta-toxin	NEM 5 mM			4/4	0/4	0/4				
C265A	NEM 0 mM							4/4	4/4	0/4
C265A	NEM 5 mM							4/4	2/4	0/4
Experiment 2[b]										
r beta-toxin	EFA 0 mM							4/4	4/4	0/4
r beta-toxin	EFA 0.05 mM						4/4	4/4	0/4	
C265H	EFA 0 mM			4/4	4/4	0/4	0/4			
C265H	EFA 0.05 mM	4/4	0/4	0/4						

The NEM or EFA-treated r β-toxin or variant toxin (0.1 ml) was injected i.v. into separate groups of four mice. Deaths occurring within 24 h were recorded.

[a]Recombinant β-toxin or C265A was incubated in 100 μl of 0.01 M phosphate buffer (pH 7.0) containing *N*-ethylmaleimide (NEM) for 30 min at 37°C.

[b]Recombinant β-toxin or C265H was incubated in 100 μl of 0.01 M phosphate buffer (pH 7.0) containing ethoxyformic anhydride (EFA) for 30 min at 37°C.

these fusion proteins with thrombin. Purified C265A, C265S, C265H and C265Y showed a single band of 35 kDa by Coomassie brilliant blue staining and immunoblotting on SDS–PAGE (Fig. 1). In addition, the mobility of E262A, Y266A, L268G, W270F and W275A was isolated as a single band with 35 kDa and the mobility of these variant toxins was identical to that of r toxin on SDS–PAGE (data not shown).

3.3. Lethal activity of variant C265A, C265S, C265H and C265Y

Lethal activity of these variant toxins at position 265 was determined in order to test if the cysteine residue is required for the activity (Table 1). Replacement of Cys-265 with alanine or serine resulted in little loss of the activity. However, the substitution of tyrosine or histidine for Cys-265 significantly diminished lethal activity by approximately 16-fold of the activity of r toxin. We have reported that treatment of β-toxin with sulfhydryl group reagents such as NEM causes drastic loss of lethal and dermonecrotic activities [9,10]. The effect of 5 mM NEM on lethal activity of r toxin and C265A was investigated (Table 2). Treatment of r toxin with the reagent reduced lethal activity by 32-fold of the activity of the untreated toxin, but the treatment of C265A led to little loss of the activity. In addition, to explore the effect of the chemical modification of the amino acid at position 265 on the activity of the toxin, r toxin and C265H were treated with 0.05 mM EFA, which is known to modify histidyl residue [16]. C265H was more sensitive to EFA than r toxin, as shown in Table 2.

To test whether replacement of the cysteine residue in r toxin causes conformational changes, r toxin and variant β-toxins (C265A, C265S, C265H and C265Y) were analyzed by CD study. The r toxin and these variant toxins showed a negative ellipticity band at approximately 215 nm and CD spectra of toxin were not different from that of these variant toxins (data not shown).

3.4. Lethal activity of variant E262A, Y266A, L268G, W270G and W275A

Hydropathy analysis of β-toxin shows that the

Table 3
Effect of W275A, Y266A and L268G on the lethal activity of β-toxin

β-Toxin	Dose of toxin (ng/mouse)			No. of mice deaths/no. of mice injected
	W275A	Y266A	L268G	
60	0			4/4
60	120			3/4
60	300			0/4
60	600			0/4
60		600		4/4
60			600	4/4

β-Toxin and Y266A, L268G or W275A were simultaneously injected into mice. Deaths occurring within 24 h were recorded.

Cys-265 residue exists between a hydrophilic region and a hydrophobic region as shown in Fig. 2. Replacement of Glu-262 in the hydrophilic region with alanine resulted in no loss of the activity. Tyr-266, Leu-268, Trp-270 and Trp-275 in the hydrophobic region were replaced with glycine, phenylalanine or alanine. Y266A, L268G and W275A showed no lethal activity under our experimental conditions, but W270F showed no loss of lethal activity. The result suggested that Tyr-266, Leu-268 and Trp-275 are essential to the activity.

To investigate the role of Tyr-266, Leu-268 and Trp-275 on lethal activity, the effect of these variant toxins on lethal activity of n toxin was determined (Table 3). Sixty ng of n toxin was mixed with various concentrations of Y266A, L268G or W275A and the mixtures were intravenously injected into mice. W275A over the concentrations 300 ng of completely inhibited lethal activity of n toxin, but 600 ng of Y266A and L268G caused no effect on the activity.

4. Discussion

Purification of β-toxin from culture supernatant fluid of *C. perfringens* type C has proved difficult for various reasons as follows: (1) the toxin is thermolabile and sensitive to proteinases, (2) the toxin is inactivated by sulfhydryl group or oxidizing agents, (3) the toxin is easy of being polymeric form, which has no activity, in the solution, and this polymeric form is difficult to be dissociated to the monomeric form or the active form [2,3]. The β-toxin gene from *C. perfringens* type B has been cloned and sequenced [11]. However, expression of the cloned toxin gene in *E. coli* caused in production of the polymeric form, which was not biologically active [11]. Steinthorsdottir et al. [12] reported that treatment of the GST-β-toxin fusion protein with Factor Xa, which hydrolyzes the bond between arginine and glycine, resulted no release of the active form of β-toxin from the fusion protein which was bound with I-E-G-R-G-I-P. In the present work, we established the isolation procedure of the active r toxin by thrombin cleavage of the GST-β-toxin fusion protein that was linked with L-V-P-R-G-S. The r toxin contained an additional Gly–Ser linked to the N-terminus of n toxin as expected, and the activity of the r toxin was as active as that of the n toxin. Accordingly, the role of the Cys-265 residue and amino acid residues near the cysteine residue was analyzed by site-directed mutagenesis using the gene of the r toxin.

The data presented here provide clear evidence that the single cysteine residue in β-toxin sensitive to sulfhydryl group reagents and oxidizing agents is not an essential residue for the lethal activity. Replacing the single cysteine residue in β-toxin with alanine or serine had no significant effect on the lethal activity. Steinthorsdottir et al. [19] reported that replacement of Cys-265 with serine resulted in no effect on the lethal activity. Such a finding is not only confined to β-toxin. Site-directed mutagenesis of *Streptococcus pneumoniae* pneumolysin, *Streptococcus pyogenes* streptolysin O and *Listeria monocytogenes* listeriolysin, which are inactivated by sulfhydryl group reagents and oxidizing agents, has revealed that the cysteine residue is not essential for the cytolytic activity of these thiol-activated toxins [20–23]. The inhibitory effect of sulfhydryl group reagents and oxidizing agents on β-toxin may be similar to that of these agents on thiol-activated toxins [20–23].

Replacement of the cysteine residue with histidine or tyrosine resulted in significant reduction in lethal activity of β-toxin. Generally it is unlikely that conformational changes in a protein are induced by a single amino acid replacement. In addition, CD spectra study indicated no difference between r toxin and these variant toxins, suggesting that the replacement of Cys-265 causes little conformational change. It therefore appears that the substitution of amino

acids, which have a larger size of side chain, for the cysteine causes reduction in the activity. Moreover, chemical modification of r toxin and C265H by NEM and EFA, respectively, led to a significant reduction in lethal activity, showing that introduction of functional groups into amino acid at position 265 diminishes the activity. It therefore appears that these inhibitory effects on lethal activity of β-toxin seems to reflect induction of steric hindrance or secondary structure changes at position 265, or both.

The thiol-activated toxins are known to have the conserved motif containing the cysteine residue (ECTGLAWEWWR) [24]. From the deduced amino acid sequence of β-toxin, the cysteine residue in β-toxin also is found to be included within the same CXXLXW sequence as thiol-activated toxins, although the sequence similarity between β-toxin and the thiol-activated toxins is very weak. It therefore is likely that the site adjacent to the cysteine residue in β-toxin is similar to that in thiol-activated toxins. Mitchell et al. [21] and Sekino-Suzuki et al. [24] reported that other substitutions within the conserved motif containing the cysteine residue in thiol-activated toxins have been shown to reduce hemolytic activity, suggesting that the site adjacent to the cysteine residue is located at a functionally important site in the toxin molecule. Replacement of Tyr-266, Leu-268 and Trp-275 in β-toxin resulted in complete loss of the activity, suggesting that these residues are important for lethal activity of β-toxin. Furthermore, the site adjacent to the cysteine residue seems to play an essential role for the activity. The thiol-activated toxins are known to be a pore-forming toxin, suggesting that the region surrounding the cysteine residue may be related to efficient oligomerization.

Hunter et al. [11] reported 28% of similarity to β-toxin to *S. aureus* α-toxin and leukocidin S. The ability of β-toxin to form polymeric form and the amino acid homology to these pore-forming toxins suggest pore formation as a potential mechanism of action for the β-toxin. Song et al. [25] reported that the membrane spanning region of *S. aureus* α-toxin participates in crucial protomer–protomer interaction as well as being important in conformational rearrangements involved in oligomer formation in the membrane bound form (position at 103 to 109, and 148 to 151). Valeva et al. [26] reported that the central molecular region of α-toxin is divided into two functional domains: the region at position 118 to 124 participates in assembly of the pre-pore, and that the region at position 126 to 140 inserts into the lipid bilayer and become part of the pore wall. These regions of the α-toxin correspond to those at positions 118 to 167 in β-toxin. On the other hand, Jursch et al. [27] reported that replacement of His-259 in the α-toxin with serine and isoleucine resulted in reduction in binding to rabbit erythrocytes, suggesting that the C-terminus of the α-toxin is involved in binding to erythrocytes. Bayley and his group [28–30] have reported that the C-terminus of the α-toxin is important for oligomerization. Walker and Byley [30] reported that replacement of Arg-200 in the α-toxin caused loss of hemolytic activity. Steinthorsdottir et al. [19] reported that a corresponding β-toxin mutant toxin (R212E) showed low lethal activity. It therefore is suggested that β-toxin forms a membrane-active oligomer. As shown in Fig. 2, the primary amino acid sequence surrounding Cys-265 in β-toxin (positions 255 to 276) is homologous to that at positions 245 to 267 in the C-terminus of α-toxin (a conserved 11-amino acid sequence), showing that the region adjacent to Cys-265 corresponds to the C-terminus of α-toxin and that Cys-265 in β-toxin corresponds to Asp-255 in the α-toxin. Walker and Bayley [30] reported that treatment of D254C and D255C (variant toxins of the α-toxin) with the sulfhydryl reagent, 4′-acetamido-4-((iodoacetyl)amino)stilbene-2,2′-disulfonate, resulted in significant reduction or complete loss of binding, oligomer formation and hemolytic activity, suggesting that C-terminus of the α-toxin is implicated in binding to cells and that the region surrounding Cys-265 in β-toxin may be required for binding to the receptor of β-toxin or formation of oligomerization.

On the basis of the hydropathy of β-toxin, the cysteine residue at position 265 is on the border between the hydrophilic region at position 256 to 264 and the hydrophobic region at position 265 to 280 (Fig. 2). Lethal activity of n toxin was inhibited by simultaneous injection of n toxin and W275A, but not by that of Y226A and L268G. W275A seems to inhibit binding of the toxin to receptor or oligomer formation, but Y226A and L268G does not. Trp-275 in β-toxin may be important for formation of efficient oligomer and Tyr-266 and Leu-268 may be required for binding to receptor or oligomer forma-

tion, suggesting that the region adjacent to the cysteine residue contributes to a functionally important structure and that the essential site for the activity of β-toxin is present in the hydrophobic region. The hydrophilic region is generally thought to be exposed toward the outside of the molecule and the hydrophobic region to be buried in the molecule. It is possible that the cysteine residue at position 265 is located at the entrance of the hydrophobic region or the cavity. It therefore appears that the introduction of functional groups by amino acid modifying agents such as NEM to the cysteine residue and replacement of the residue with amino acid with a large size of side chain such as tyrosine and histidine results in steric hindrance to pore formation of β-toxin or the access of the toxin to receptor. A cytotoxicity model with cultured cells should be more suitable to address this question. This is now the subject of investigation in our laboratory.

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